Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1159–1163, March 1983
Biochemistry

An intermediate step in translocation of lipopolysaccharide to the outer membrane of *Salmonella typhimurium*

(O antigen/immunoelectron microscopy)

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Contributed by Mary Jane Osborn, August 16, 1982

ABSTRACT **Evidence for transient localization of newly synthesized lipopolysaccharide at the periplasmic face of the inner membrane has been obtained by immunoelectron microscopic techniques. *Salmonella typhimurium galE* mutants in which O-antigen synthesis is dependent on addition of exogenous galactose were employed, and the distribution and fate of pulse-synthesized O antigen was examined by indirect ferritin labeling with anti-O-antigen IgG of spheroplasts prepared by treatment with lysozyme/EDTA. O-reactive lipopolysaccharide appeared rapidly at the exposed periplasmic face of the inner membrane after addition of galactose and was rapidly depleted upon termination of the pulse. Control experiments showed that secondary redistribution of lipopolysaccharide from outer membrane did not occur under the conditions employed for spheroplast formation and immunolabeling, and the pulse–chase kinetics were consistent with those expected for an intermediate in translocation of lipopolysaccharide to the outer membrane. In addition, undecaprenol-linked O antigen was detectable at the periplasmic face of the inner membrane within 30 sec after addition of galactose to a *galE* deep rough double mutant, and it accumulated stably in that location. The mutation in synthesis of the lipopolysaccharide core in the deep rough strain prevents transfer of O-antigen chains from undecaprenol phosphate to lipopolysaccharide. The result suggests that attachment of O antigen to lipopolysaccharide occurs on the extracytoplasmic side of the inner membrane and supports the conclusion that lipopolysaccharide is translocated to the outer membrane from the periplasmic, rather than the cytoplasmic, face of the inner membrane.**

Previous studies on the assembly of lipopolysaccharide into the outer membrane of *Salmonella typhimurium* have established that this molecule is synthesized in the inner, cytoplasmic membrane and is subsequently translocated to the outer membrane (1). This translocation is rapid and irreversible (1, 2), and it appears to occur at a limited number of contact sites between the inner and outer membranes, the zones of adhesion (2–4). However, the molecular mechanisms of translocation and integration into outer membrane are still poorly understood.

Current models of translocation assume that synthesis of lipopolysaccharide is initiated at the inner, cytoplasmic face of the inner membrane and impose a formal requirement for transposition of the molecule across two bilayers in order to achieve its known asymmetric distribution (5, 6) in the external leaflet of the outer membrane. The possibility that newly synthesized lipopolysaccharide might be transiently localized at the external, periplasmic face of the inner membrane as an intermediate in translocation was suggested by early immunoelectron microscopic studies of Shands (7, 8). By using immunoferritin techniques, extensive labeling with lipopolysaccharide-specific antibody was found on the exposed periplasmic face of the cytoplasmic membrane as well as on both faces of the outer membrane in penicillin spheroplasts of *S. typhimurium*. However, it was later demonstrated by Muhlradt and Golecki (5) that lipopolysaccharide is restricted to the outer face of outer membrane in murein–outer membrane preparations treated with lysozyme at 0°C, but it undergoes rapid redistribution with loss of asymmetry upon exposure of the membrane preparation to 37°C. The significance of Shands' results was thus unclear. More recently, Takamiya *et al.* (9) have also observed labeling of the periplasmic face of the inner membrane (and both sides of outer membrane) after postembedding staining of intact cells with ferritin-conjugated anti-lipopolysaccharide antibody. However, in this case protease treatment of the sectioned preparations was required for effective labeling.

The studies reported here were undertaken to reinvestigate by immunoelectron microscopy the occurrence of lipopolysaccharide at the periplasmic face of the inner membrane and its potential role in translocation under conditions in which redistribution artifacts could be both minimized and recognized. The evidence strongly supports the hypothesis that newly synthesized lipopolysaccharide appears transiently at the periplasmic face of the inner membrane as an intermediate stage in translocation to outer membrane. In addition, the results suggest that terminal steps in lipopolysaccharide biosynthesis are localized at this site.

MATERIALS AND METHODS

Bacterial Strains and Media. *S. typhimurium* LT2 and a mutant of this strain that lacks UDP-galactose 4-epimerase (G30) have been described (10, 11). Strain G30A, a derivative of G30, produces a heptose-deficient lipopolysaccharide (12). Cultures were grown at 32°C or 37°C in proteose peptone/beef extract medium (PPBE) (13) supplemented with 0.2 mM galactose where indicated. PPBE has been found to contain trace amounts of galactose (14). This could be eliminated by incubation of fresh PPBE for 1 hr with G30 cells (4–5 × 10^9 per ml) that had been previously grown to exponential phase and harvested. The medium was then cleared of cells by centrifugation and resterilized by passage through a 0.45-μm-pore-diameter Millipore filter.

Immunoelectron Microscopy. LT2 O-antigen-specific antisera were prepared in New Zealand White rabbits as described (15). IgG was purified from whole serum by passage through a column of Sepharose CL-4B covalently coupled with staphylococcal protein A (Sigma). Adsorbed IgG was eluted with 0.1 M glycine·HCl buffer, pH 3.0, and fractions were collected into tubes containing 75 μl of 1 M Tris base. Specificity of this IgG for O antigen was demonstrated electron microscopically by absence of ferritin labeling in cells or spheroplasts of O-negative cells. Ferritin-conjugated goat anti-rabbit IgG was obtained

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Abbreviation: PPBE, proteose peptone/beef extract.

from Miles-Yeda (Rehovot, Israel).

All experimental procedures were carried out at 0°C. The samples, both cells and spheroplasts formed by treatment with lysozyme/EDTA (1), were prefixed for 20 min with 0.2% glutaraldehyde in 25 mM sodium phosphate, pH 7.0. Glycine (0.1 M) was then added to discharge excess free aldehyde. Samples were centrifuged, incubated with IgG specific for O antigen for 30 min, and then treated with ferritin-conjugated goat anti-rabbit IgG for 30 min. All samples were washed three times after each of these two incubations. They were then suspended in phosphate-buffered 4% (wt/vol) glutaraldehyde for 3 min, followed by addition of phosphate-buffered 2% OsO_4. The samples were pelleted by centrifugation and allowed to stand for 1 hr at 0–4°C. The pellets were dehydrated and embedded in Epon (16). Thin sections were mounted on copper grids, stained with bismuth (17) or uranyl acetate/lead citrate (18), and examined with a Hitachi HU-11E electron microscope.

RESULTS

Distribution of O-Antigen-Containing Lipopolysaccharide in Spheroplasts. In preliminary experiments lysozyme/EDTA spheroplasts of wild-type *S. typhimurium* LT2 were prepared at 0°C as described (19) and subjected to indirect ferritin-labeling with O-antigen-specific IgG and ferritin-conjugated anti-IgG. In confirmation of the results of Shands (7, 8) and Takamiya *et al.* (9), substantial ferritin labeling was observed at the exposed periplasmic face of the inner membrane in addition to the external face of the outer membrane (data not shown). However, no ferritin was detected at the periplasmic face of the outer membrane. Similar results were obtained with a *galE* mutant, G30, grown in the presence of galactose to permit synthesis of complete immunoreactive lipopolysaccharide. Spheroplasts exposed to preimmune serum were free of ferritin, as were cells and spheroplasts of strain G30 grown under conditions nonpermissive for O-antigen synthesis (galactose-free medium).

Control for Secondary Redistribution of Lipopolysaccharide. In order to exclude the possibility that lipopolysaccharide detected at the periplasmic face of the inner membrane arose by artifactual redistribution during experimental manipulations, advantage was taken of the conditional lipopolysaccharide phenotype of the *galE* mutant, G30. One culture was grown in the presence of galactose for four generations, then switched to galactose-free medium for one generation. Thus all immunoreactive O-antigen-containing lipopolysaccharide was at least one generation old and would be expected to reside exclusively in the outer membrane. A second, parallel culture was exposed to galactose only in the final generation of growth such that all immunoreactive O antigen was of recent origin. Lysozyme/EDTA spheroplasts were prepared at 0°C and lightly prefixed with glutaraldehyde, and O-antigen-containing lipopolysaccharide was visualized by indirect ferritin labeling (Fig. 1). Cells that had received galactose during the final generation of growth (Fig. 1*a*) showed ferritin at the external surfaces of both inner and outer membranes, as described above. In contrast, the exposed inner membrane of cells that had been deprived of galactose for one generation and contained only old O antigen was entirely free of ferritin (Fig. 1*b*). Thus no significant redistribution of immunoreactive lipopolysaccharide integrated into the outer membrane during previous growth had occurred during spheroplast formation and immunolabeling procedures. However, when these spheroplasts were warmed to 37°C for 1 min before glutaraldehyde prefixation the ferritin labeling pattern indicated substantial redistribution of old O-antigenic lipopolysaccharide to the periplasmic faces of both the outer membrane and the inner membrane (Fig. 2).

Kinetics of Appearance and Subsequent Translocation to Outer Membrane. The rate of appearance of newly synthesized O-reactive lipopolysaccharide at the periplasmic face of the inner membrane was determined after addition of galactose to a growing culture of G30. Ferritin-labeled O antigen could be detected at the periplasmic face of the inner membrane as early as 30 sec after addition of galactose (see below), and the density of labeling reached an apparent steady state within 2 min (Fig. 3*a*). It should be noted that control cells grown in PPBE medium without added galactose showed significant ferritin on the

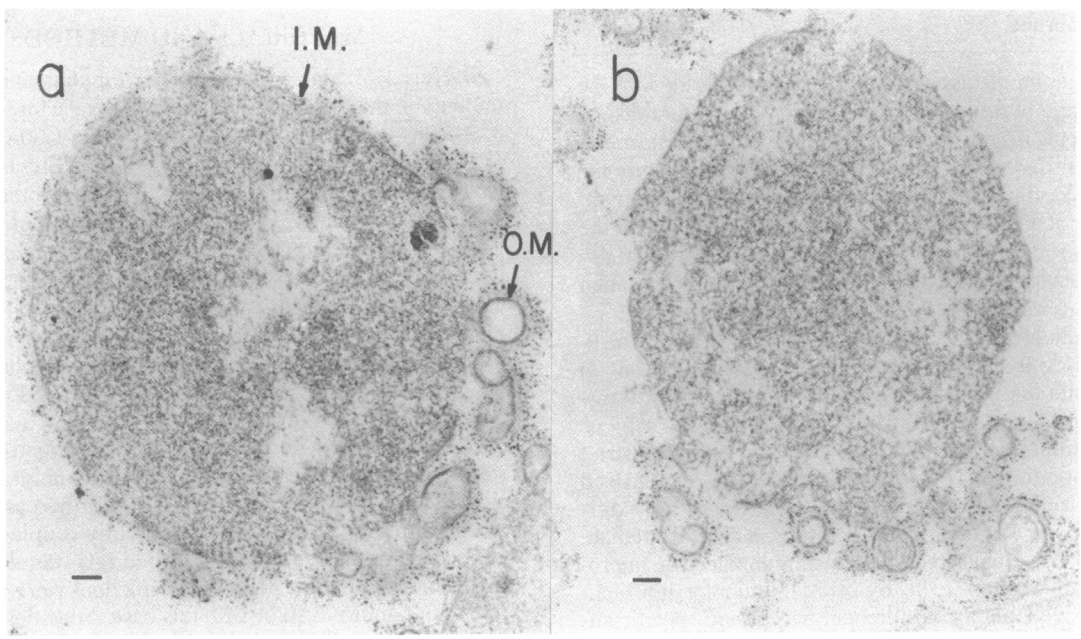

FIG. 1. Distribution of lipopolysaccharide in spheroplasts. (*a*) G30 cells were grown in PPBE medium for four generations before addition of 0.2 mM galactose and the cells were harvested after one more generation. Cells were processed as described in the text. (*b*) G30 cells were grown with galactose (0.2 mM) for four generations, washed, and suspended in PPBE minus galactose for the final generation of growth. (×37,000.) In this and subsequent figures the bars represent 0.1 μm. O.M., outer membrane; I.M., inner membrane.

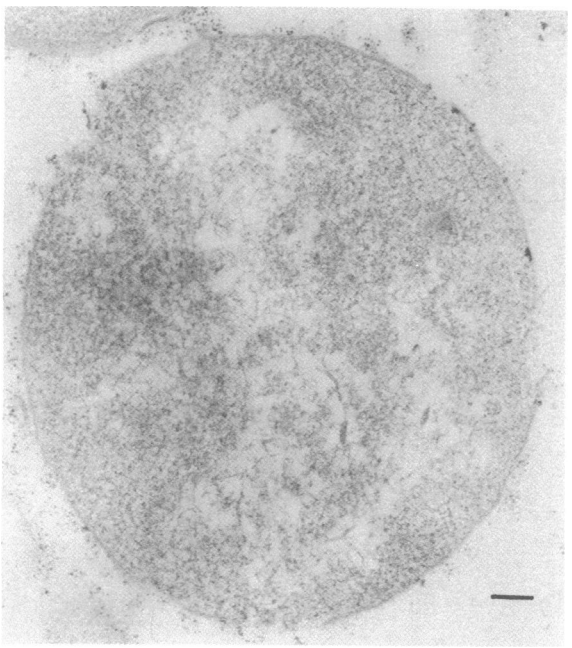

FIG. 2. Redistribution of lipopolysaccharide in G30 warmed to 37°C. Spheroplasts from sample *b* of Fig. 1, were warmed to 37°C for 1 min before prefixation and processing. (×56,000.)

outer membrane although the inner membrane was unlabeled. This proved to result from variable trace contamination of the medium by galactose and could be suppressed by use of preconditioned medium (see *Materials and Methods*).

Evidence that lipopolysaccharide detected at the periplasmic face of the inner membrane represents an intermediate in translocation was provided by immunoelectron microscopic pulse–chase experiments. Ten-fold-concentrated suspensions of G30 were pulsed with galactose (40 μM) for 5 min at 32°C and the galactose was chased by dilution into fresh medium containing 5 mM glucose. Glucose effectively blocks galactose transport and incorporation into lipopolysaccharide. [^{14}C]Galactose was included in the pulse, and parallel samples were taken for electron microscopy and sucrose density gradient separation of inner and outer membranes (1) in order to establish kinetics of overall translocation from inner to outer membrane under these experimental conditions. Immunoferritin labeling showed progressive loss of pulse-synthesized O-reactive lipopolysaccharide from the periplasmic face of the inner membrane during the chase period. Ferritin labeling of exposed inner membrane was greatly reduced after 5 min of chase (Fig. 3*b*) and was essentially absent by 10 min (Fig. 3*c*). These chase kinetics correlated well with the overall rate of translocation of ^{14}C-labeled lipopolysaccharide to outer membrane (data not shown). However, it was not possible to establish by these experiments that the immunoreactive lipopolysaccharide that disappeared from the periplasmic face of the inner membrane quantitatively reappeared in the outer membrane.

Localization of Undecaprenol-Linked O-Antigen Intermediates. The repeating unit of the O-antigen chain is synthesized and polymerized via a series of membrane-bound intermediates linked to the carrier lipid, undecaprenol phosphate (20), and the polymer chain is then transferred to the independently synthesized lipopolysaccharide core as the final step in assembly of the complete molecule (21). Evidence for rapid appearance and accumulation of undecaprenol-phosphate-linked O antigen at the periplasmic face of the inner membrane was obtained by use of a deep rough derivative of G30, strain G30A. This mutant

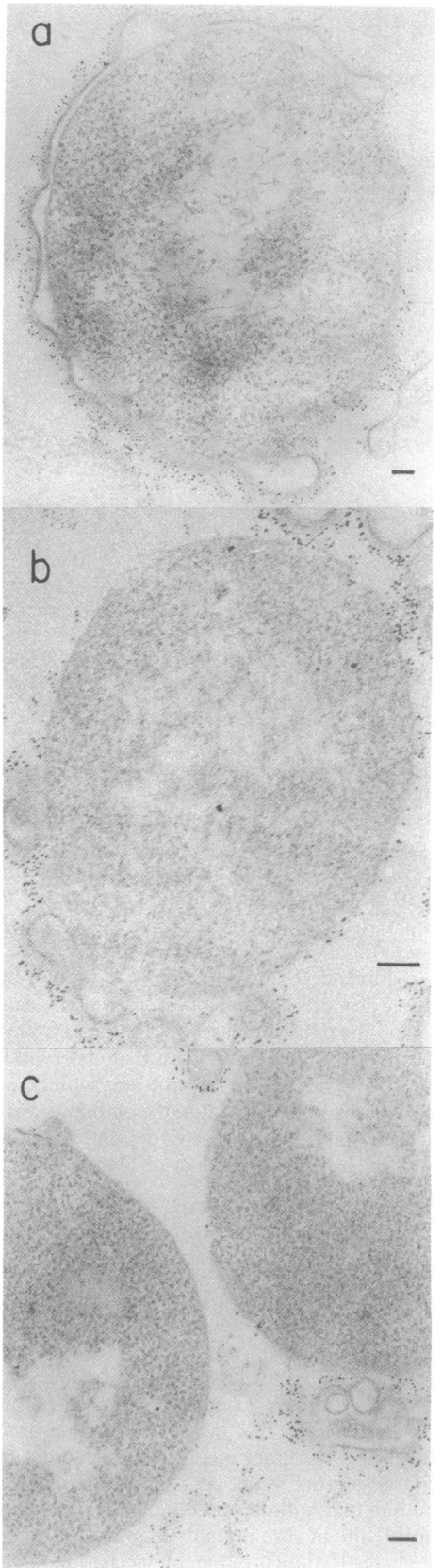

FIG. 3. Kinetics of galactose pulse and chase. (*a*) Two minutes after addition of galactose to G30. (×38,000.) Chase was initiated by 20-fold dilution of the culture into PPBE containing 5 mM glucose, and samples were removed and mixed into ice and 2,4-dinitrophenol (2.5 mM) at intervals for harvest and spheroplast preparation (1). (*b*) Five-minute chase. (×61,000.) (*c*) Ten-minute chase. (×35,000.)

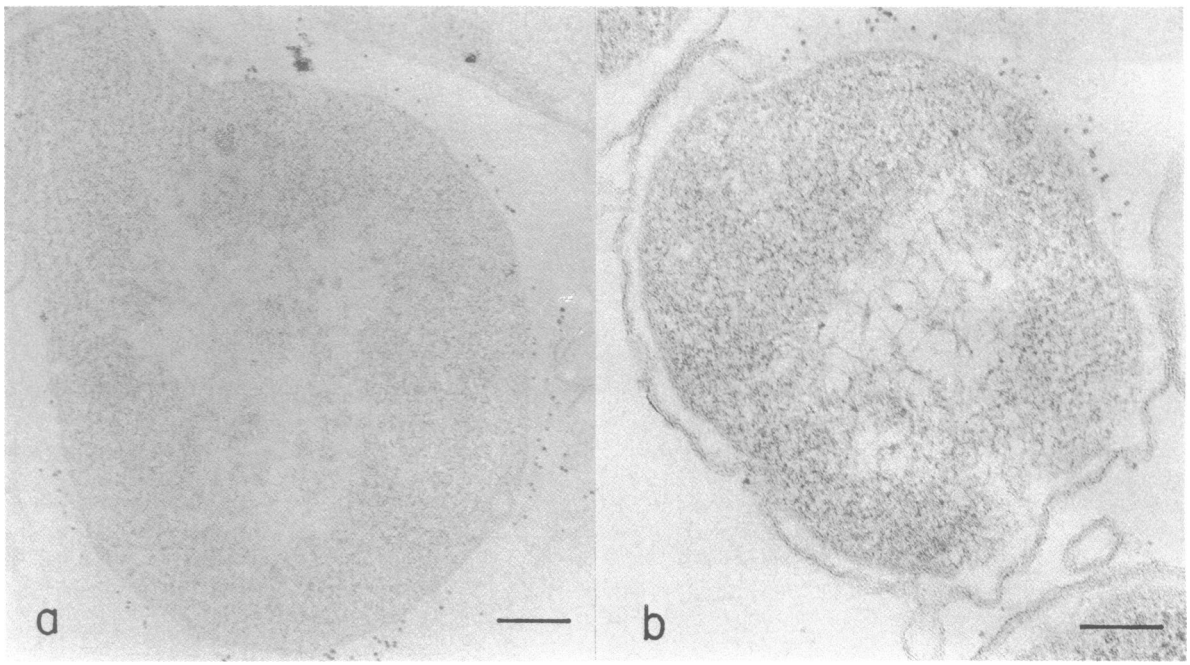

FIG. 4. Appearance of undecaprenol-linked polymeric O antigen at the periplasmic face of the inner membrane. Strain G30A was exposed to 0.2 mM galactose. Samples were removed at intervals and chilled in the presence of 2,4-dinitrophenol. (*a*) Thirty seconds after addition of galactose. (×97,000.) (*b*) Two minutes after addition of galactose. (×108,000.)

produces an incomplete heptose-deficient core lipopolysaccharide that lacks O-antigen attachment sites, but the strain retains the ability to synthesize undecaprenol-linked O antigen in the presence of exogenous galactose. Ferritin-labeled O antigen was evident on the inner membrane of many spheroplasts as soon as 30 sec (Fig. 4*a*) after addition of galactose to the culture and increased in density up to 2 min (Fig. 4*b*). No significant increase in labeling after 2 min was observed. Accumulation of lipid-linked O-antigen polymer is limited by the low concentration of undecaprenol phosphate in the inner membrane (21), and the density of ferritin labeling in G30A at steady state was correspondingly lower than that observed in G30 or wild-type cells. In addition, no ferritin was found on the outer membrane and the labeling of inner membrane was stable to chase (data not shown), as expected for the nontranslocated, nonlipopolysaccharide end product of O-antigen synthesis in this strain.

DISCUSSION

The present studies confirm earlier observations of others (7–9) on the presence of lipopolysaccharide at the periplasmic face of the inner membrane of *S. typhimurium* and establish that this lipopolysaccharide does not arise by secondary redistribution from the outer membrane, but consists of newly synthesized or nascent molecules that are rapidly depleted from this location during chase. Evidence that lipopolysaccharide occupies the periplasmic face of the inner membrane as an obligatory intermediate in translocation is not yet complete. However, the additional finding that undecaprenol-linked O antigen also accumulates at this site in core mutants implies that attachment of O-antigen chains to the core lipopolysaccharide takes place at the periplasmic face and supports the conclusion. Our results also confirm the findings of Muhlradt and Golecki (5) on the asymmetric transmembrane orientation of lipopolysaccharide in the outer membrane and the occurrence of a temperature-dependent redistribution of outer membrane lipopolysaccharide after disruption of the underlying murein layer. The latter phenomenon seems to involve frank release of lipopolysaccharide from the outer membrane and nonspecific readsorption to available bilayer surfaces, but the nature of the perturbation and the presumptive role of the murein cytoskeleton in stabilization of outer membrane lipopolysaccharide are not clear.

Current models of lipopolysaccharide translocation derive from immunoelectron microscopic studies of Muhlradt and coworkers (2) and postulate transfer to the outer membrane at discrete sites of contact between inner and outer membrane, the zones of adhesion described by Bayer (22). However, the molecular architecture of zones of adhesion has remained elusive, and a variety of structures can be imagined that lead to quite different models of molecular events in translocation. Possibilities include passive lateral diffusion of lipopolysaccharide from the cytoplasmic leaflet of the inner membrane to the external leaflet of the outer membrane at sites of continuity between the two bilayers or direct transfer from cytoplasmic to external face at regions of fusion between inner and outer membrane (23). The present results are not consistent with such models, and they suggest a more specific and complex mechanism as follows: (*i*) syntheses of core lipopolysaccharide and O-antigen chains are initiated separately at the cytoplasmic face of the inner membrane; (*ii*) core lipopolysaccharide and lipid-linked intermediates of O-antigen synthesis are independently transposed to the periplasmic face, where (*iii*) attachment of O antigen to the core (and perhaps polymerization of O-antigen chains) takes place; (*iv*) intermembrane transfer of lipopolysaccharide from the periplasmic face of the inner membrane to outer membrane then occurs at sites of close apposition of the two bilayers, as postulated by Muhlradt *et al.* (2). Evidence to be presented elsewhere has shown that the overall process is dependent on maintenance of both membrane potential and intracellular ATP pools, and indirect evidence (24) suggests that some step(s) may require facilitation.

The possible role of carrier lipid, undecaprenol phosphate, in transposition of peptidoglycan intermediates across the cytoplasmic membrane for extracytoplasmic polymerization was recognized early by Strominger and colleagues (25), and the application of this idea to lipopolysaccharide assembly was dis-

cussed by Nikaido in 1973 (26). However, the assumption that synthesis of core lipopolysaccharide and O-antigen intermediates is initiated on the cytoplasmic side of the membrane has not been experimentally tested, and the possibility that the entire biosynthetic membrane takes place at the periplasmic face requires consideration. The latter would presumably require transport of cytoplasmically synthesized substrates, such as nucleotide sugars, to the periplasmic site of utilization. The problem is analogous to that of transmembrane synthesis of dolichol-linked saccharide units in endoplasmic reticulum (27, 28). Preliminary efforts to determine the transmembrane orientation of lipopolysaccharide biosynthetic enzymes by protease sensitivity and nucleotide sugar accessibility indicate that the CMP-ketodeoxyoctulosonate:lipid A ketodeoxyoctulosonate transferase system is indeed localized at the cytoplasmic face of the inner membrane, but these experiments have given ambiguous results for galactosyl pyrophosphorylundecaprenol synthetase (unpublished experiments). Our initial attempts to detect immunoreactive O-antigen intermediates at the cytoplasmic face of the inner membrane by electron microscopy have been technically unsatisfactory, and the topology of O-antigen synthesis remains to be resolved.

Electron microscopy was carried out at the University of Connecticut Health Center central Electron Microscope Facility (Dr. T. J. MacAlister, Director). We are grateful to Dr. MacAlister for much helpful advice and discussion. The research was supported by National Institutes of Health Research Grant AI 08650 and National Institutes of Health Training Grant GM 07407 (to C.A.M.).

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